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Characterization of *Alpha*-Adrenergic Receptors in Guinea-pig Vas Deferens by [^{3}H]Dihydroergocryptine Binding

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SUMMARY

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[^{3}H]Dihydroergocryptine binds to membrane sites on guinea-pig vas deferens, which have the characteristics expected of α-adrenoreceptors. Specific binding is rapid, reversible and saturable, with a maximal occupancy of 190 fmol [^{3}H]dihydroergocryptine bound/mg protein. Saturable binding occurs to a single population of high affinity sites (K_D = 1.55 nM) with no evidence for cooperative interactions. [^{3}H]Dihydroergocryptine binding is markedly pH and temperature sensitive. While Na^+ and K^+ do not affect binding, maximal [^{3}H]dihydroergocryptine binding observed in the presence of Mg^{2+} is antagonized by increasing concentrations of Ca^{2+}. α-Adrenoreceptor agonists and antagonists of several classes inhibit binding to vas deferens membranes. Catecholamines show marked stereospecificity in relation to inhibition of binding of [^{3}H]dihydroergocryptine. The potency for displacement of [^{3}H]dihydroergocryptine by phenylethylamine agonists is identical to their agonistic potency in eliciting contractile responses of the isolated guinea-pig vas deferens. Some discrepancies between binding and physiological affinities of imidazoline α-adrenoreceptor ligands were observed and discussed. There is close agreement between dissociation constants of azapetine and dihydroergocryptine, as estimated from radioligand binding and antagonism of physiological responses. As no other biogenic amine receptors binds [^{3}H]dihydroergocryptine, there is no interference in using this radioligand to study α-adrenoreceptor mechanisms in guinea-pig vas deferens.

INTRODUCTION

In recent years, the availability of radioligands labeled to high specific activity has provided the means for identification and characterization of numerous membrane-bound receptors for neurotransmitters. Among these radioligands, the α-adrenergic antagonist, [^{3}H]DHE[1], has been used to identify α-adrenergic binding sites in membranes prepared from rabbit uterus (1), rat parotid acinar cells (2), rat brain (3, 4) and human platelets (5). In these studies, [^{3}H]DHE binding appeared to be rapid and reversible, with the labeled sites exhibiting the specificity characteristic of α-adrenergic receptors. α-Adrenoreceptor agonists and antagonists competed with [^{3}H]DHE for its binding site with the stereospecificity and order of potency paralleling their physiological actions.

The guinea-pig vas deferens is characterized by a dense, predominantly sympathetic innervation (6) and a narrow, 20 nm wide synaptic cleft (7). Electrophysiological (8)

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[1] The abbreviation used is [^{3}H]DHE, [^{3}H]dihydroergocryptine.

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and pharmacological evidence (9, 10) suggests that norepinephrine is the transmitter mediating excitatory contractile responses in vas deferens, via α-adrenoreceptor stimulation. The presynaptic innervation of guinea-pig vas deferens also contains a significant α-adrenoreceptor component, mediating feedback control of sympathetic neurotransmitter secretion (11). These factors have contributed to the importance of the guinea-pig vas deferens as a valuable pharmacological test preparation for assaying the potencies of numerous α-adrenoreceptor agonists and antagonists (12, 13).

The present study was undertaken to identify and characterize the α-adrenergic receptor of guinea-pig vas deferens utilizing the radioligand [^{3}H]DHE. In this paper, we present a detailed analysis of the kinetics, affinity and specificity of [^{3}H]DHE binding to membranes of guinea-pig vas deferens. The correlation between affinities of α-adrenoreceptor ligands for the membrane binding site with their physiological potencies demonstrates the usefulness of the vas deferens as a suitable model for investigating α-adrenoreceptor regulation.

Preliminary accounts and demonstrations of the results have been given previously (14).

MATERIALS AND METHODS

Radioligand and compounds. [^{3}H]Dihydroergocryptine (24-28 Ci/mmole) was obtained from New England Nuclear. Preparation and purification of this radioligand have been previously described (1). The compound was stable when stored in ethanol at −20°. A fresh stock solution was made immediately before use by adding appropriate aliquots of [^{3}H]DHE to an aqueous solution containing 5 mM HCl and 10% ethanol.

Among the compounds used in this study were (−)norepinephrine, (−)epinephrine, (−)phenylephrine hydrochloride, dopamine hydrochloride, ergotamine tartrate, dihydroergotamine tartrate, ergocryptine and ergocristine sulfate from Sigma. (+)Norepinephrine bitartrate, (+)epinephrine bitartrate and (−)α-methyl norepinephrine were obtained from Sterling-Winthrop. Other drugs used in the investigation included phentolamine hydrochloride (Ciba-Geigy), dihydroergocryptine methane sulfonate (Sandoz), phenoxybenzamine hydrochloride (Smith, Kline and French), propranolol hydrochloride (Ayerst), and azapetine phosphate (Hoffman-LaRoche). Serotonin creatinine sulfate monohydrate and histamine dihydrochloride were obtained from Aldrich.

Stock solutions of phenoxybenzamine hydrochloride, ergotamine tartrate, dihydroergotamine tartrate, ergocryptine, ergocristine sulfate, (−)α-methyl norepinephrine and dihydroergocryptine methane sulfonate were prepared in an aqueous solution containing 10% ethanol and 5 mM HCl. Serial dilutions of these stocks were made in water, as were stock solutions of all other compounds tested. Ascorbate (0.1%) was added to all stocks and dilutions of the catecholamines to retard spontaneous oxidation.

Membrane preparations. Smooth muscle membranes were prepared from frozen vasa deferentia of mature guinea-pigs (400–600 g weight) obtained from Pel-Freeze Biologicals. Initial experiments demonstrated that membranes prepared from frozen vas deferens gave binding results quantitatively similar to fresh guinea-pig vas deferens membranes. [^{3}H]Dihydroergocryptine binding sites of frozen vas deferens remained stable when the tissues were stored at −90°. Tissues were used within six weeks of freezing.

Vasa deferentia were thawed, cleaned and stripped of surrounding connective tissue and vasculature. The tissues were minced, with 3–4 vasa deferentia pooled per 3 ml ice-cold buffer (0.25 M sucrose, 5 mM Tris and 1 mM $MgCl_2$, pH 7.4) and homogenized for 30 sec using a Brinkmann Polytron (PT-10) at maximal setting. The homogenate was then centrifuged at 500 × g for 10 min at 4°. The presence of $MgCl_2$ (1 mM) in the homogenizing buffer was essential for adequate sedimentation of nuclei, unbroken cells and other nonmembraneous particles during this initial centrifugation. The supernatants from this spin were pooled and centrifuged at 100,000 × g for 30 min at 4°. The resulting pellet was suspended in 4 ml ice-cold incubation buffer

(50 mM Tris and 10 mM $MgCl_2$, pH 7.5) and centrifuged at 100,000 × g for 30 min. The final pellet was resuspended in incubation buffer to yield 1–1.5 mg of protein per milliliter for subsequent use in the binding assay. Protein concentrations were determined by the method of Lowry *et al.* (15).

Binding assay. The standard binding assay was performed in 150 μl of incubation buffer containing approximately 150 μg protein, 2 nM [^{3}H]DHE and varying concentrations of other drugs or their solvent. The binding reaction was initiated upon addition of protein, and incubations allowed to proceed for 15 min at 25° in a shaking water bath. The assay was performed in the dark to minimize [^{3}H]DHE decomposition. Incubations were terminated by dilution with 2 ml of incubation buffer (25°), followed by rapid filtration of the mixture through Whatman GF/C glass fiber filters. After rinsing the incubation tube twice, the filters were washed with 12 ml of incubation buffer (25°). Dilution of membranes and filter washing required less than 20 sec. Filters were allowed to dry, placed into liquid scintillation vials containing 10 ml of a scintillation cocktail (Triton X-100 33%, toluene 63% and Liquifluor 4%) and counted at an efficiency of 32–37%.

Specific receptor binding of [^{3}H]DHE is defined as the difference between total radioactivity bound and nonspecific radioligand binding. Nonspecific [^{3}H]DHE binding was considered to be radioligand bound in the presence of excess α-antagonist phentolamine (10 μM). Specific binding was consistently 75% of the total counts bound to membrane protein. About 1% of the radioligand added was nonspecifically bound to the glass filters in the absence of protein, but was not displaced by any of the compounds tested.

Contractile measurements. Correlative physiological data for the compounds assayed by radioligand binding were obtained by recording their effects on vas deferens contractile activity. Guinea-pigs weighing 350–450 g were sacrificed by cervical dislocation, and the vasa deferentia were excised and stripped of connective tissue. Tissues were mounted in a 10 ml organ bath containing physiological salt solution (NaCl 115.7 mM; KCl 5.9 mM; $CaCl_2$ 1.4 mM; $MgCl_2$ 1.0 mM, $NaHCO_3$ 27.5 mM and dextrose 24.7 mM). The bath was aerated with 95% O_2–5% CO_2, pH was 7.35 and temperature was maintained at 35°. Each strip was initially placed under 1.5 *g* passive tension and allowed to equilibrate for 1 hr. Isometric contractions were measured by a force-displacement transducer (Grass FT-03) and recorded on a polygraph (Grass Model 79).

At the outset of each experiment, responsiveness of each vas deferens was measured to a test concentration of epinephrine (20 μM). Strips responding with a developed tension less than 2.5 g were discarded. In all experiments, at least 8 min was allowed to elapse between administration of agonists to minimize the possibility of α-adrenoreceptor desensitization (16, 17). α-Antagonist potencies were determined from their effects on epinephrine-induced contractions of the vas deferens. Antagonists were pre-equilibrated with the tissue for 30 min.

Statistical differences were evaluated by Student's *t*-test.

RESULTS

Saturability of [^{3}H]DHE binding. Specific binding of [^{3}H]DHE to membranes prepared from guinea-pig vas deferens was saturable, with a maximal occupancy of 190 fmol [^{3}H]DHE bound/mg protein (Fig. 1A). Half-maximal binding occurred at 1.55 nM [^{3}H]DHE, and provides an estimate of the equilibrium dissociation constant (K_D) of the radioligand for its binding site. Analysis of the concentration-saturation data by a Scatchard plot (18) yields a straight line, suggesting the existence of a single population of binding sites (Fig. 1B). A K_D of 1.2 nM, determined from the slope of this line, agrees with the dissociation constant value obtained at half-maximal saturation (Fig. 1A). The number of [^{3}H]DHE binding sites on vas deferens membranes (n = 190 fmol/mg protein) calculated from the abscissa-intercept of the Scatchard plot (Fig. 1B) is identical to the maximal occupancy obtained from the concentration-saturation curve (Fig. 1A). Plotting the data according to the Hill equation yielded a straight line with a slope of 0.92 (Fig. 1C), suggesting

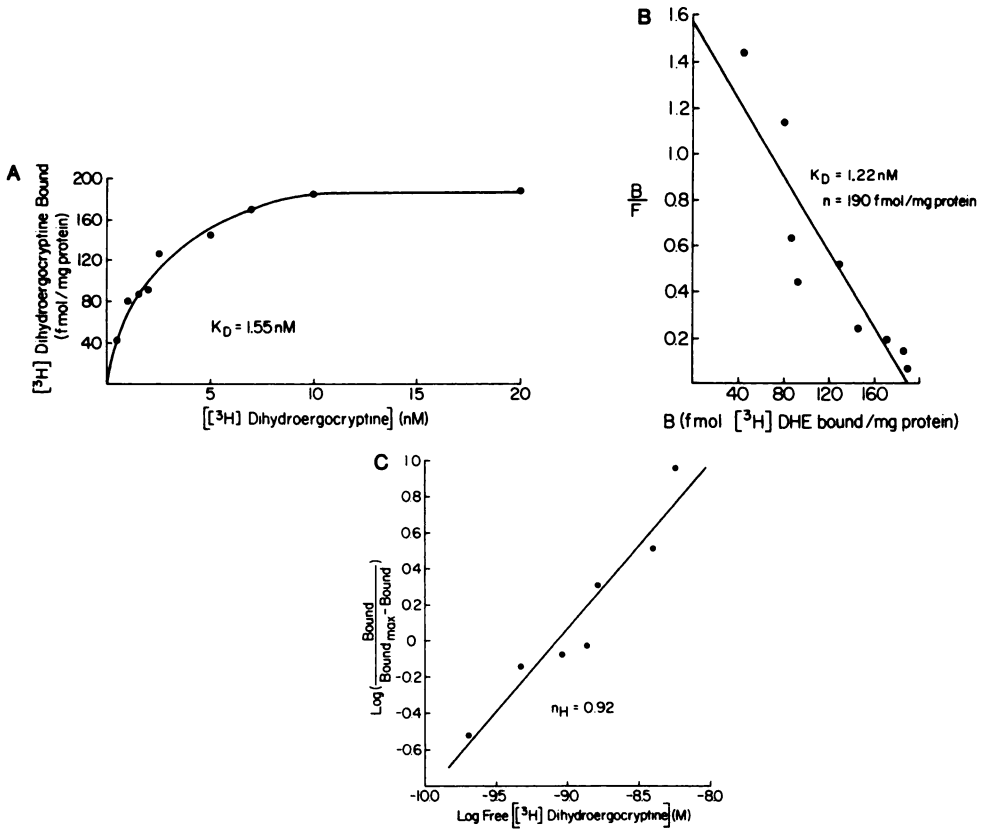

FIG. 1. *Specific binding of [^{3}H]dihydroergocryptine to guinea-pig vas deferens membranes*

Panel A) As a function of the [^{3}H]dihydroergocryptine concentration. Vas deferens membranes were incubated with the indicated concentration of [^{3}H]dihydroergocryptine for 15 min at 25°, and specific binding was determined as described under MATERIALS AND METHODS. Each value represents the mean of 3–6 determinations. Panel B) Scatchard plot of [^{3}H]dihydroergocryptine binding to guinea-pig vas deferens membranes. The ratio of bound/free [^{3}H]dihydroergocryptine is plotted against the concentration of bound radioligand. The slope of the plot, $-1/K_D$, was determined by linear regression analysis ($r = -0.91$). The number of binding sites, n, is calculated from the x-intercept. Each value is the mean of 3–6 determinations. Panel C) Hill plot of [^{3}H]dihydroergocryptine binding to guinea-pig vas deferens membranes. The values for [[^{3}H]dihydroergocryptine] (Bound, Bound_{max} and Free) were obtained from Figure 1A. The slope of the plot, n_H, was determined by linear regression analysis ($r = 0.95$). Each value is the mean, n = 3–6.

the absence of any cooperative interactions among [^{3}H]DHE binding sites.

Kinetics of [^{3}H]DHE binding. [^{3}H]DHE binding to guinea-pig vas deferens membranes was rapid (t 1/2 = 2.5 min), and equilibrium was reached by 15 min at 25° (Fig. 2A). A pseudo-first order rate constant (k_{ob}) of 0.202/min for the association reaction was obtained (Fig. 2B). Taking the reversibility of [^{3}H]DHE binding (Fig. 3A) and the radioligand concentration into account, a second order rate constant of association (k_1) was determined from the equation (1):

$$k_1 = \frac{(k_{ob} - k_2)}{[\text{DHE}]} = 7.6 \times 10^7/\text{M/min}$$

where k_2 is the rate constant for dissociation and [DHE] refers to the concentration of [^{3}H]DHE. Binding was reversible (t 1/2 = 13.5 min) at 25° (Fig. 3A), with dissociation of [^{3}H]DHE from its binding site following first-order kinetics. Thus, a rate constant for dissociation (k_2) of 0.05/min was determined from the slope of a first-order rate plot (Fig. 3B). A kinetically derived estimate of the equilibrium dissociation constant was obtained from the ratio of the

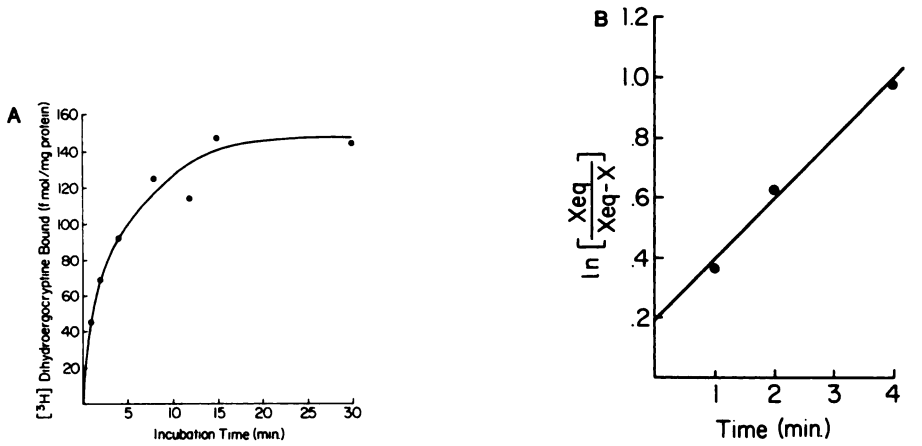

FIG. 2. *Binding of [^{3}H]dihydroergocryptine to guinea-pig vas deferens membranes as a function of time*
Panel A) [^{3}H]Dihydroergocryptine (2 nM) was incubated with membranes for the indicated times at 25°, and specific binding determined as described in MATERIALS AND METHODS. Each value is the mean of 9 determinations. Panel B) Pseudo-first order kinetic plot of initial [^{3}H]dihydroergocryptine binding to guinea-pig vas deferens membranes. X refers to amount of [^{3}H]dihydroergocryptine bound at time t, while X_{eq} is the amount of [^{3}H]dihydroergocryptine bound at equilibrium. The slope (k_{ob}) of the line, determined by linear regression analysis (r = 0.99), represents the observed rate constant for the pseudo-first order reaction. Each value is the mean of 9 determinations.

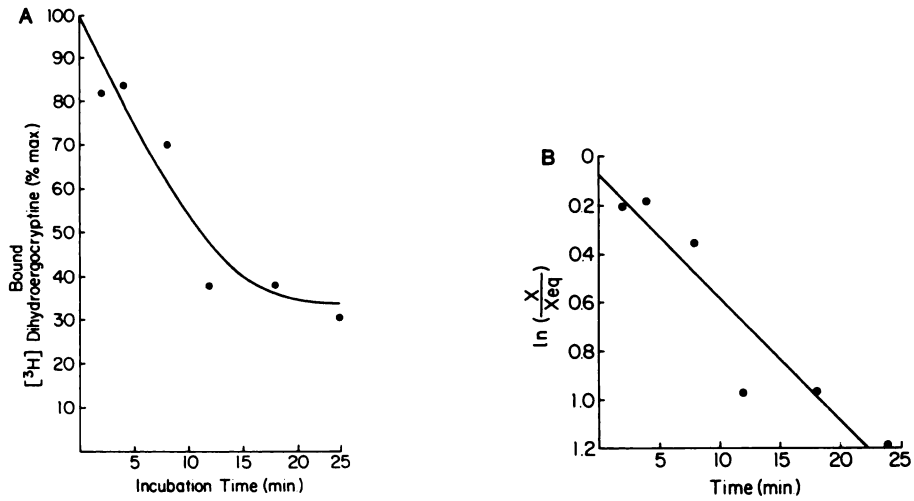

FIG. 3. *Dissociation of [^{3}H]dihydroergocryptine from guinea-pig vas deferens membranes*
Panel A) Membranes were preincubated with [^{3}H]dihydroergocryptine (2 nM) at 25° for 15 min, followed by addition of an excess of phentolamine (10 μM) corresponding to time, t = 0. Specific binding was determined at subsequent times as described in MATERIALS AND METHODS. 100% bound corresponds to specific binding of [^{3}H]dihydroergocryptine (2 nM) at t = 0. Each value is the mean of 9 determinations. Panel B) First-order kinetic plot of [^{3}H]dihydroergocryptine dissociation from guinea-pig vas deferens membranes. X_{eq} refers to the amount of specific binding at t = 0 (maximum bound), while X is the amount bound at the indicated times after addition of excess phentolamine (10 μM). The slope of the line, determined by linear regression analysis (r = −0.94), corresponds to the first-order rate constant of dissociation. Each value is the mean of 9 determinations.

two rate constants: $K_D = k_2/k_1 = 0.66$ nM. As this value is in approximate agreement with the K_D obtained from the concentration-saturation curve (Fig. 1A), it validates the assumption that [^{3}H]DHE binding to vas deferens membranes was measured un-

der steady-state conditions.

[^{3}H]DHE binding conditions. Several factors relevant to establishing optimal binding conditions for [^{3}H]DHE were investigated. Varying the pH of the incubation buffer markedly altered radioligand binding. Specific binding of [^{3}H]DHE to vas deferens membranes was optimal between pH 7.0 and 7.5 (Fig. 4), decreasing sharply under more acidic or basic incubation conditions. Varying the ionic composition of the incubation buffer influenced the extent of [^{3}H]DHE binding. Although the presence of monovalent cations, NaCl (100 mM) or KCl (10 mM), in the standard buffer did not affect specific binding (Table 1), addition of $CaCl_2$ to the assay medium resulted in a concentration-dependent (2.5–10 mM) decrease in [^{3}H]DHE binding. This reduction in specific binding was manifested by reduced total binding, with no effect on nonspecific binding. The removal of $MgCl_2$ (10 mM) from the incubation buffer caused a 25% reduction in binding, which could not be restored to control levels by $CaCl_2$ (2.5–10 mM) (Table 1). This suggests that Ca^{2+} cannot substitute for Mg^{2+} in the Tris-buffered incubation medium. Vas deferens membranes suspended in buffer containing the detergents Triton X-100 (0.05%) or Lubrol-PX (1–5 w/w protein) no longer exhibited specific binding. Thus, detergent pretreatment appeared to destroy [^{3}H]DHE binding to the *alpha*-adrenoreceptor.

The specific binding of [^{3}H]DHE to vas deferens membranes was temperature sensitive. Although increasing the incubation temperature (35°) did not affect the association rate or maximum specific binding, decreased temperatures greatly affected the kinetics of association. At 15°, the ap-

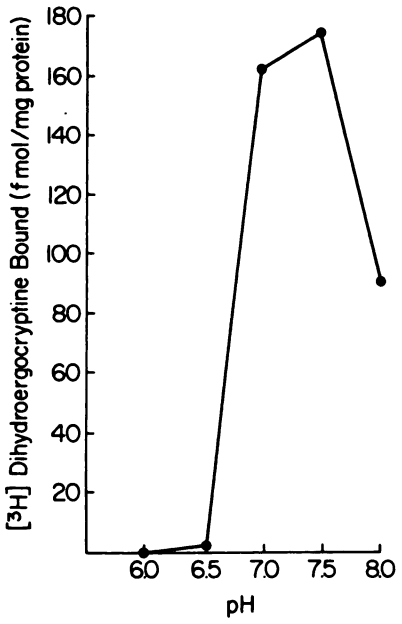

FIG. 4. *Specific binding of [^{3}H]dihydroergocryptine to guinea-pig vas deferens membranes as a function of pH of the incubation buffer*

Membranes, suspended in incubation buffer of indicated pH, were incubated with [^{3}H]dihydroergocryptine (2 nM) at 25° for 15 min. Specific binding was determined as described in MATERIALS AND METHODS, except that the incubation was terminated and filters washed with incubation buffer of the indicated pH. Each value is the mean of 3 determinations.

TABLE 1

Effects of incubation buffer ionic conditions on specific [^{3}H]dihydroergocryptine binding to guinea-pig vas deferens membranes

Guinea-pig vas deferens membranes suspended in incubation buffer (pH 7.5) containing the ions indicated were incubated with [^{3}H]dihydroergocryptine for 15 min at 25°. Specific radioligand binding was determined as described in MATERIALS AND METHODS. Each value is the mean ± standard error of 3-6 determinations.

Incubation buffer	Specific [^{3}H]dihydroergocryptine binding
	(% control)
Tris (50 mM), $MgCl_2$ (10 mM)	100 ± 8.2
Tris (50 mM), $MgCl_2$ (10 mM) + NaCl (100 mM)	114.8 ± 7.4
Tris (50 mM), $MgCl_2$ (10 mM) + KCl (10 mM)	102.4 ± 3.5
Tris (50 mM), $MgCl_2$ (10 mM)	
+ $CaCl_2$ (2.5 mM)	73.1 ± 6.6
+ $CaCl_2$ (5 mM)	66.2 ± 10.5[a]
+ $CaCl_2$ (10 mM)	56.2 ± 8.3[b]
Tris (50 mM)	75.6 ± 10.2
Tris (50 mM)	
+ $CaCl_2$ (2.5 mM)	69.7 ± 23.9
+ $CaCl_2$ (5 mM)	78.4 ± 26.6
+ $CaCl_2$ (10 mM)	57.6 ± 8.2[b]

[a] $p < 0.05$ compared with control: Tris (50 mM), $MgCl_2$ (10 mM).

[b] $p < 0.02$ compared with control: Tris (50 mM), $MgCl_2$ (10 mM).

proximate t 1/2 of association was increased to 13.5 min (Fig. 5). After 60 min incubation, the amount of [^{3}H]DHE bound at 15° and 5° was respectively only 80% and 24% of control (Fig. 5).

Specificity of [^{3}H]DHE binding and correlation with physiological responses. α-Adrenoreceptor agonists of the phenylethylamine class competed with [^{3}H]DHE for vas deferens membrane binding with the potency order: (−)epinephrine ≥ (−)norepinephrine > (−)phenylephrine > (−)α-methyl norepinephrine (Fig. 6). These four agonists exhibited an identical order of potency in their ability to elicit contractile responses of isolated guinea-pig vas deferens (Fig. 7). The equilibrium dissociation constants for these phenylethylamines derived from the radioligand binding studies and from their physiological responses under the conditions studied were in close agreement (Table 2). The presence of cocaine (50 μM) shifts the physiological concentration-response curve to norepinephrine 17-fold to the left (Table 2). (−)Epinephrine and (−)norepinephrine were over 100-fold more potent than their corresponding (+)stereoisomers in displacing [^{3}H]-DHE (Fig. 6). The low affinity of the (+)catecholamines for the [^{3}H]DHE binding site precluded an accurate determination of a dissociation constant. Thus, inhibition of [^{3}H]DHE binding by catecholamines is markedly stereospecific and demonstrates an α-adrenoreceptor specificity, which has been characterized for numerous

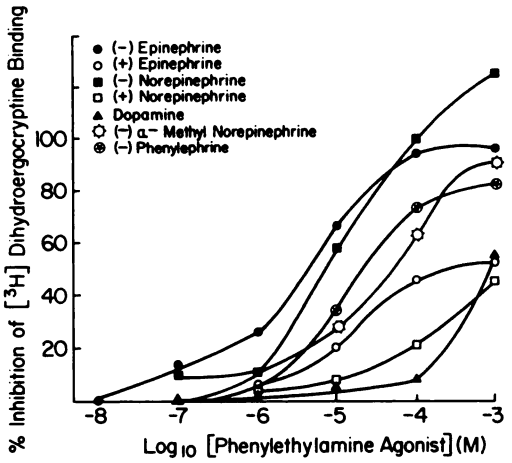

FIG. 6. *Displacement of [^{3}H]dihydroergocryptine binding by phenylethylamine agonists*

Vas deferens membranes were incubated for 15 min at 25° with [^{3}H]dihydroergocryptine (2 nM) in the presence of indicated concentrations of (−)epinephrine (●), (+)epinephrine (○), (−)norepinephrine (■), (+)norepinephrine (□), dopamine (▲), (−)phenylephrine (⊛), or (−)α-methyl norepinephrine (☼). Determination of specific binding is described in MATERIALS AND METHODS. "100% inhibition" is defined as inhibition of binding by phentolamine (10 μM). Each value is the mean of 4 determinations.

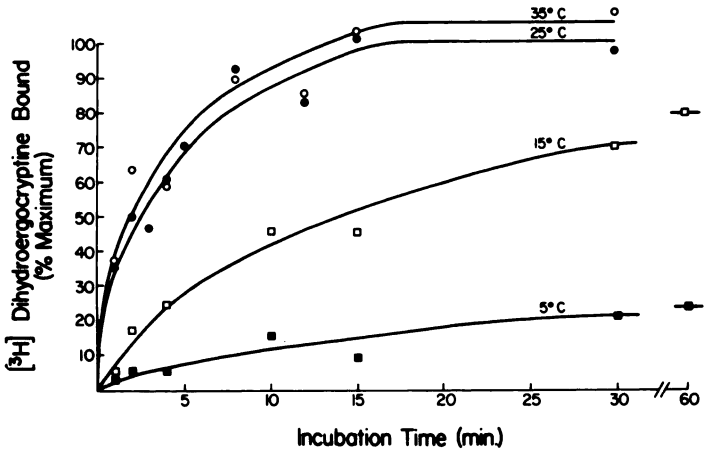

FIG. 5. *Temperature dependence of [^{3}H]dihydroergocryptine binding to guinea-pig vas deferens membranes as a function of time*

[^{3}H]Dihydroergocryptine (2 nM) was incubated with membranes for the indicated times at the following temperatures: 5° (■), 15° (□), 25° (●) and 35° (○). Specific binding was assayed as described under MATERIALS AND METHODS. Each value is the mean of 3 determinations.

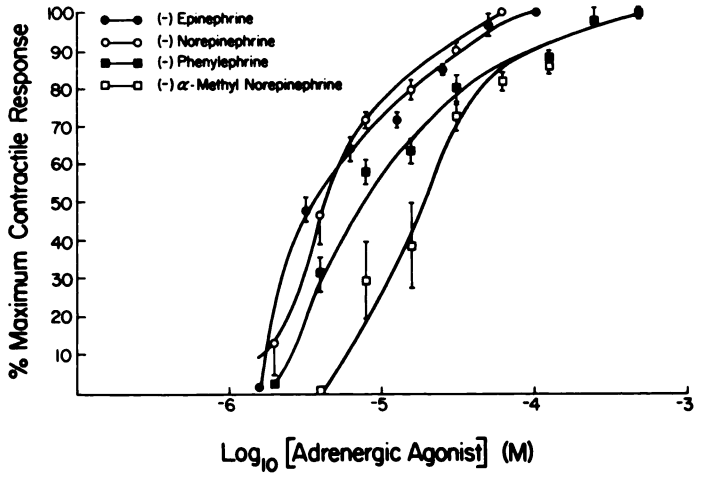

FIG. 7. *Concentration-response curves for α-adrenoreceptor agonist-induced contractions of guinea-pig vas deferens*

Isolated vasa deferentia were mounted in a 10 ml organ bath, bathed in physiological salt solution at 35° and isometric contractions recorded to exogenously administered agonists. The curves shown are: (−)epinephrine (●), (−)norepinephrine (○), (−)phenylephrine (■) and (−)α-methyl norepinephrine (□). Each point represents the mean ± S. E. of 4–5 determinations.

catecholamine-mediated physiological responses (19).

Dopamine and histamine elicit contractile activity in the isolated guinea-pig vas deferens (12, 13), and along with serotonin, have been shown to displace [^{3}H]DHE from rabbit uterine membranes (1). Dopamine had a low affinity (K_D = 349 μM) for the [^{3}H]DHE binding site of guinea-pig vas deferens (Fig. 6 and Table 2). At concentrations up to 1 mM, neither histamine or serotonin caused any displacement of radioligand.

α-Adrenoreceptor antagonists were two to three orders of magnitude more potent than α-agonists (Table 2) in displacing [^{3}H]DHE from vas deferens membrane sites. The ergot alkaloids demonstrated the highest affinity for [^{3}H]DHE binding sites, with a potency order: dihydroergocryptine > dihydroergotamine ≥ ergotamine > ergocristine > ergocryptine (Table 2). The K_D for dihydroergocryptine was identical to the equilibrium dissociation constant for [^{3}H]DHE obtained from the concentration-saturation curve (Fig. 1A), suggesting that the bioactivity of the radioligand is equivalent to the unlabeled antagonist. The other α-antagonists assayed, displayed an order of potency: phentolamine > phenoxybenzamine > oxymetazoline > azapetine (Fig. 8), with a wider range of affinities for the [^{3}H]DHE binding site than displayed by the ergot alkaloids (Table 2). The β-receptor antagonist, propranolol, was 1000-fold less potent than the weakest α-antagonist, azapetine, indicating that [^{3}H]DHE does not label a β-adrenoreceptor site (Fig. 8 and Table 2).

The physiological potencies of the α-receptor blockers were determined from their abilities to antagonize epinephrine-induced contractions of isolated vas deferens. Phentolamine and azapetine (0.5 μM) caused parallel rightward shifts of the concentration-response curve to epinephrine (Fig. 9). The physiological dissociation constant for antagonism of α-mediated contractility by azapetine agrees closely with its K_D for inhibition of [^{3}H]DHE binding (Table 2). On the other hand, the physiological K_D for phentolamine when determined at either 500 or 50 nM is 36-fold higher than the K_D determined by radioligand binding (Table 2). The imidazoline oxymetazoline, generally classified as an α-agonist (17, 19), failed to elicit consistent contractile responses of the isolated guinea-pig vas deferens. Instead, oxymetazoline demonstrated competitive antagonism of contractile re-

TABLE 2

Dissociation constants (K_D) for ligand displacement of [^{3}H]dihydroergocryptine binding to vas deferens membranes and effects on vas deferens contractility

Guinea-pig vas deferens membranes were incubated with [^{3}H]dihydroergocryptine in the presence of each ligand, and specific binding was evaluated, as described in MATERIALS AND METHODS. The equilibrium dissociation constant, K_D, for each compound was determined from the equation (20):

$$K_D = \frac{EC_{50}}{1 + \frac{[DHE]}{K_{DHE}}}$$

where EC_{50} is the ligand concentration causing half-maximal displacement of [^{3}H]dihydroergocryptine binding, [DHE] refers to the concentration of radioligand in the assay (2 nM), and K_{DHE} is the dissociation constant (1.55 nM) determined from the equilibrium saturation data (Fig. 1A). Each value is the mean of four determinations.

Isometric contractions of isolated vas deferens to exogenously administered agonists were obtained as described in MATERIALS AND METHODS. K_D for contractions of vas deferens by α-agonists is defined as the half-maximal (EC_{50}) concentration obtained from the curves in Figure 8. Antagonist potencies were evaluated by their effects on epinephrine-induced contractions. Dissociation constants for antagonists were determined from the equation (21):

$$K_D = \frac{[\text{Antagonist}]}{\text{Dose Ratio} - 1}$$

where the dose ratio was obtained from the EC_{50} concentrations of epinephrine in the absence and presence of antagonist (Fig. 10). Each value is the mean of 3–5 determinations.

Compound	K_D for inhibition of [^{3}H]-dihydroergocryptine binding	K_D for contractility
	(M)	(M)
α-agonists		
(−) Epinephrine	1.66×10^{-6}	3.4×10^{-6} 2.5×10^{-6}[a]
(−) Norepinephrine	3.06×10^{-6}	4.5×10^{-6} 2.6×10^{-7}[a]
(−) Phenylephrine	10^{-5}	7×10^{-6}
(−) α-Methyl Norepinephrine	1.97×10^{-5}	2×10^{-5}
α-antagonists		
Phentolamine	6.55×10^{-9}	2.35×10^{-7}
Oxymetazoline	4.37×10^{-8}	1.1×10^{-6}
Azapetine	1.22×10^{-7}	3.33×10^{-7}
Phenoxybenzamine	1.31×10^{-8}	
Dihydroergocryptine	1.55×10^{-9}	1.1×10^{-9}[b]
Ergocryptine	7.42×10^{-9}	
Ergotamine	2.18×10^{-9}	
Dihydroergotamine	2.1×10^{-9}	
Ergocristine	2.97×10^{-9}	
Other ligands		
Dopamine	3.49×10^{-4}	
Propranolol	1.05×10^{-4}	

[a] In the presence of 50 μM cocaine.
[b] K_D is given for 10 nM dihydroergocryptine.

sponses to epinephrine, with K_D determined to be 1.1 μM, at each of two concentrations tested (Fig. 10). As with phentolamine, the physiological K_D for oxymetazoline is 33-fold higher than the K_D value determined from inhibition of [^{3}H]DHE binding (Table 2). Thus, the affinity of imidazolines for the [^{3}H]DHE binding site does not correspond to their antagonistic potency against α-adrenoreceptor-mediated contractile responses in guinea-pig vas deferens.

Dihydroergocryptine antagonizes vas deferens contractile responses to epinephrine in a competitive manner, causing a shift to the right, of the epinephrine concentration-response curve without reduction of the maximal response (Fig. 11). From the dose-ratio, calculated at the EC_{50} concentration, K_D's ranging between 1.1–0.17 nM (Table 2) were calculated from experiments at two concentrations of dihydroergocryptine according to Furchgott's equation (21, 22). Thus, the K_D for dihydroergocryptine, determined from radioligand binding studies and physiological measurement, is in relatively close agreement. The physiological antagonism exerted by dihydroergocryptine appears to be specific for α-adrenoreceptors, since contractions in response to carbachol and histamine do not appear to be influenced. However, after the vas deferens has been in contact with dihydroergocryptine for a prolonged period, as in the determination of physiological antagonism to epinephrine, the effects of this antagonist do not appear to be readily reversible by prolonged periods of washing.

DISCUSSION

The extensive use of the guinea-pig vas deferens as a pharmacological test preparation for investigating the effects of α-adrenoreceptor agonists and antagonists (8–14, 16, 17) makes it desirable to define this tissue as a potential model for studying the molecular mechanisms underlying α-adrenergic receptor regulation. The affinity of [^{3}H]DHE for the vas deferens α-adrenoreceptor (Fig. 1A) is on the same order of magnitude as its affinity for rabbit uterine (1), rat parotid (2) and brain (3, 4) membrane binding sites. In common with other tissues (1–5), the guinea-pig vas deferens

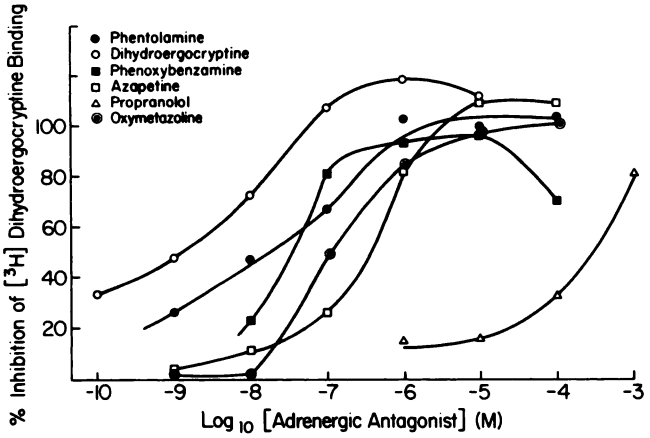

FIG. 8. *Displacement of [^{3}H]dihydroergocryptine binding by adrenergic antagonists*

Vas deferens membranes were incubated for 15 min at 25° with [^{3}H]dihydroergocryptine (2 nM) in the presence of indicated concentrations of phentolamine (●), dihydroergocryptine (○), phenoxybenzamine (■), azapetine (□), propranolol (△) or oxymetazoline (⊙). Determination of specific binding is described in MATERIALS AND METHODS. "100% inhibition" is defined as inhibition of binding by phentolamine (10 μM). Each value represents the mean of 4 determinations.

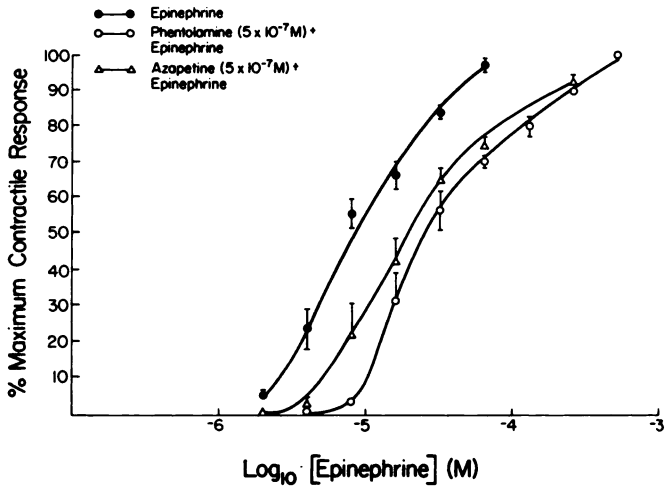

FIG. 9. *The effects of α-adrenoreceptor blocking drugs on the concentration-response curve for (−)epinephrine-induced contractions of guinea-pig vas deferens*

Isometric contractions to epinephrine were recorded from isolated tissues mounted in an organ bath under conditions described in MATERIALS AND METHODS. Vas deferens were exposed to each antagonist for 30 min. The curves shown include: control (−)epinephrine (●), and in the presence of phentolamine (0.5 μM) (○), and azapetine (0.5 μM) (△). Each point represents the mean ± S. E. for 3 determinations, except for the control (−)epinephrine, which is the composite of 10 determinations.

contains only one population of high-affinity [^{3}H]DHE binding sites (Fig. 1B), which exhibit no cooperative interactions (Fig. 1C). The binding of [^{3}H]DHE to vas deferens membranes is rapid (Fig. 2) and reversible (Fig. 3), with association and dissociation kinetics similar to [^{3}H]DHE binding to rabbit uterine (1) and rat parotid (2) membranes. The kinetically-derived equilibrium dissociation constant agrees with the K_D value determined at half-maximal binding (Fig. 1A), corroborating the accuracy of the estimate of [^{3}H]DHE affinity for the α-adrenoreceptor.

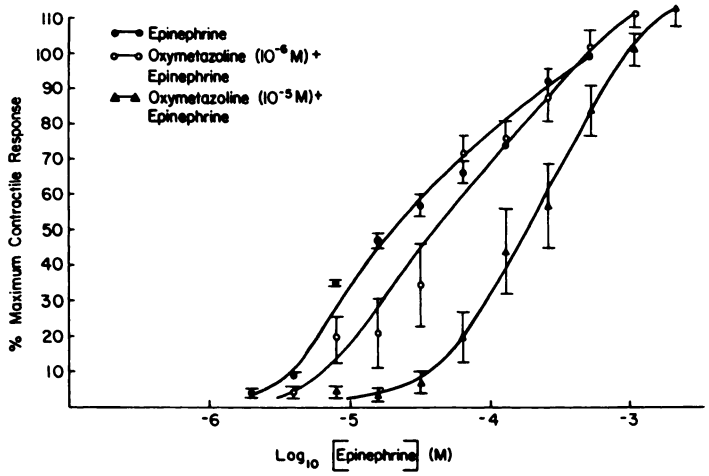

FIG. 10. *The antagonism of (−)epinephrine-induced contractions of guinea-pig vas deferens by oxymetazoline*

Isometric contractions to epinephrine were recorded from isolated tissues mounted in an organ bath under conditions described in MATERIALS AND METHODS. Vas deferens were exposed to oxymetazoline for 30 min. The curves shown include: control (−)epinephrine (●), and in the presence of oxymetazoline (1 μM) (○) and (10 μM) (▲). Each point represents the mean ± S. E. for 4 determinations.

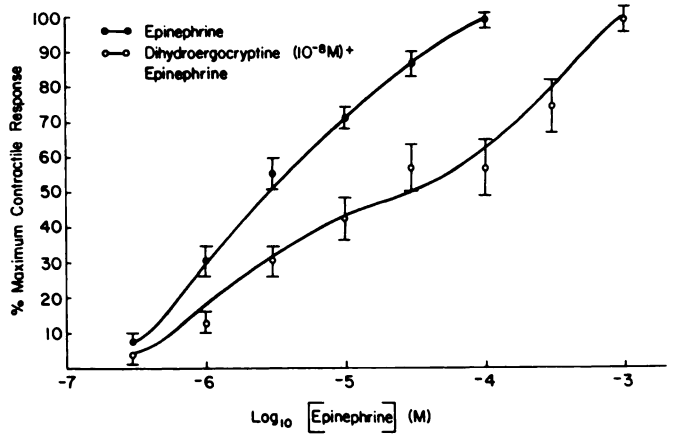

FIG. 11. *The antagonism of (−)epinephrine-induced contractions of guinea-pig vas deferens by dihydroergocryptine*

Isometric contractions to epinephrine, in the presence of cocaine (50 μM), were recorded from isolated tissues mounted in an organ bath under conditions described in MATERIALS AND METHODS. Vas deferens were exposed to dihydroergocryptine for 30 min. The curves shown include: control (−)epinephrine (●), and in the presence of dihydroergocryptine (10 nM) (○). Each point represents the mean ± S. E. for 9 determinations.

Optimal binding of [^{3}H]DHE observed at physiological pH (Fig. 4) may reflect the charge distribution over the ergot alkaloid and its binding site, allowing for the most favorable interaction between drug and receptor. [^{3}H]DHE binding to guinea-pig vas deferens membranes appears to be optimal in the presence of Mg^{2+} (Table 1). That Ca^{2+} cannot substitute for Mg^{2+}, in this regard, implies a degree of specificity in the divalent ion contribution to binding of this radioligand. The inhibition of [^{3}H]DHE binding to vas deferens by Ca^{2+} (Table 1) contrasts with the lack of effect by Ca^{2+} on [^{3}H]DHE binding to rabbit uterine membranes (23). This differential effect of Ca^{2+} may be due to different membrane site characteristics between the two species.

Nevertheless, it appears that [^{3}H]DHE binding to guinea-pig vas deferens membranes is selectively influenced by divalent, but not monovalent cations. The marked temperature-dependence of [^{3}H]DHE binding kinetics (Fig. 5) contrasts with the relative insensitivity to temperature of β-adrenoreceptor association with the antagonist [^{3}H]dihydroalprenolol (24). While no comparable data exists for the temperature-sensitivity of [^{3}H]DHE binding in other tissues, the kinetics and magnitude of [^{3}H] catecholamine agonist binding to calf brain α-adrenoreceptors is also markedly temperature dependent (25). The decreased binding to vas deferens membranes at 5° and 15° (Fig. 5) implies reduced accessibility of the α-adrenoreceptor to [^{3}H]DHE, due perhaps to decreased biomembrane fluidity at these lowered temperatures and an influence of membrane lipids on the availability of the receptor to ligands.

Radioligand displacement by a series of α-agonists (Fig. 6) and antagonists (Fig. 8) demonstrates the specificity of [^{3}H]DHE binding to the α-adrenergic receptor of guinea pig vas deferens. The stereoselectivity of catecholamine inhibition of binding (Fig. 6) parallels the greater physiological potency of (−)catecholamines in eliciting vas deferens contractions than their (+)stereoisomers (26). The guinea-pig vas deferens α-adrenoreceptor exhibits a greater apparent stereoselectivity toward catecholamines than rabbit uterine (1) and rat brain (3, 4) α-adrenergic receptors. The potency order for displacement of [^{3}H]-DHE by phenylethylamine agonists (Fig. 6) is identical to their agonistic potency in eliciting contractile responses of the isolated vas deferens (Fig. 7). Under conditions of neuronal reuptake blockade by cocaine, the physiological dissociation constant value for norepinephrine is decreased (Table 2). Nevertheless, the observed potency order for the phenylethylamine agonists, as determined by radioligand binding and physiological responses, remains characteristic of expected interactions with α-adrenoreceptors (1–5, 19, 26).

The lack of [^{3}H]DHE displacement by histamine and serotonin is in contrast with the ability of these amines to displace radioligand from rabbit uterine membranes (1). In rat brain membranes, serotonin has an even greater affinity for [^{3}H]DHE binding sites than the catecholamine agonists (3). The potency difference between (−)catecholamine agonists and dopamine in displacing [^{3}H]DHE from vas deferens membranes (Fig. 6) is much greater than for rabbit uterus (1) and rat brain (3, 4). Thus, specific binding of [^{3}H]DHE to guinea-pig vas deferens is subject to much less interference by other receptors in comparison with other tissues (1–4), emphasizing the value of this tissue for the study of α-adrenoreceptor mechanisms.

The potency order for displacement of [^{3}H]DHE from vas deferens membranes by α-adrenoreceptor antagonists (Fig. 8) agrees with the order for other tissues (1–4). The specificity of [^{3}H]DHE for the α-adrenoreceptor is verified by the weak potency of the β-antagonist propranolol (Fig. 8). The ergot alkaloids exhibit the highest affinity for the binding site (Table 2), and consistent with their physiological potencies (19) the dihydrogenated ergot alkaloids are more potent than the unsaturated analogues. The close agreement between dissociation constants of azapetine and dihydroergocryptine (Table 2), as estimated from radioligand binding (Fig. 8) and antagonism of physiological responses (Figs. 9 and 11), validates the hypothesis that [^{3}H]DHE binds to the α-adrenoreceptor of guinea-pig vas deferens. The only uncharacteristic aspect of the action of dihydroergocryptine as an α-adrenoreceptor antagonist is our observation that it is not readily reversible by washing after use in experiments on physiological antagonism. It is important that this lack of reversibility is characteristic of situations in which intact tissue is exposed to the drug under conditions (35°, oxygenation, long time periods) of greatest vulnerability to chemical and/or metabolic degradation (27). The possibility that degradation products may accumulate in the vas deferens, accounting for the difference between the membrane and the intact tissue behavior, is under investigation.

The imidazoline derivatives, oxymetazoline and phentolamine, are potent inhibi-

tors of [^{3}H]DHE binding to vas deferens membranes. Oxymetazoline has generally been classified as an α-adrenoreceptor agonist in most systems, e.g., vascular smooth muscle (28, 29). Our results, however, demonstrate that oxymetazoline is a competitive antagonist in guinea-pig vas deferens, resembling the typical imidazoline α-antagonist phentolamine in its ability to antagonize epinephrine-induced contractions of this tissue (Fig. 10). Imidazoline antagonists also exhibit a 30 to 35-fold lower potency in antagonizing epinephrine-induced contractions compared with inhibition of [^{3}H]DHE binding to vas deferens (Table 2). Newman *et al.* (5) reported a similar discrepancy between the ability of phentolamine to inhibit [^{3}H]DHE binding and antagonize epinephrine-induced inhibition of PGE_1-stimulated adenylate cyclase in platelet lysates. While the reason for the dissociation between physiological antagonism and [^{3}H]-DHE displacement by imidazolines is not apparent, several suggestions may be proposed. Ruffolo *et al.* (30) demonstrated that binding of the α-antagonist [^{3}H]dihydroazapetine to rat vas deferens is inhibited by imidazolines, but paradoxically enhanced by (−) stereoisomers of phenylethylamines. It was proposed that imidazolines bind to a site different from, but interacting with, the catecholamine recognition site of the α-adrenoreceptor (30). Although all physiologically-active α-ligands inhibited [^{3}H]-DHE binding in guinea-pig vas deferens, the existence of two distinct, but interacting α-adrenoreceptor sites may underly the discrepancy between K_D values for phentolamine and oxymetazoline as determined from contractile and [^{3}H]DHE binding data (Table 2). An additional contribution to this discrepancy could also reside in tissue uptake and/or metabolism of imidazolines, thereby altering their assumed free concentration in the vicinity of the α-adrenoreceptor.

In conclusion, the data indicate that [^{3}H]DHE binds to a membrane site on guinea-pig vas deferens which has binding characteristics expected of an α-adrenergic receptor (1–5). The data demonstrate relatively close agreement between the potency order for inhibition of [^{3}H]DHE binding to guinea-pig vas deferens membranes by numerous α-adrenoreceptor ligands and their order of potency determined from physiological responses by the same tissue (Table 2). This would suggest that [^{3}H]DHE binds to the α-adrenergic receptor of guinea-pig vas deferens.

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